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### Drug metabolism in human and rat intestine

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*Document Version*

Publisher's PDF, also known as Version of record

*Publication date:*

2007

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

van de Kerkhof, E. G. (2007). *Drug metabolism in human and rat intestine: an 'in vitro' approach*. s.n.

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## Chapter 3

**Characterization of rat small intestinal and colon precision-cut slices as an *in vitro* system for drug metabolism and induction studies**

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*Adapted from: DMD 33: 1613-20, 2005*

## Abstract

The aim of this study was to characterize rat small intestinal and colon tissue slices as a tool to study intestinal metabolism and to investigate gradients of drug metabolism along the intestinal tract as well as drug-induced inhibition and induction of biotransformation. Tissue morphology and the intestinal mucus layer remained intact in small intestinal and colon slices during 3 hours of incubation, while the rate of metabolism of three model compounds (7-hydroxycoumarin, 7-ethoxycoumarin and testosterone) appeared constant. Phase I and phase II metabolic gradients, decreasing from stomach towards colon were shown to be clearly different for the model compounds used. Furthermore, the observed slice activities were similar or even higher compared with the literature data concerning metabolism of *in vitro* intestinal systems. Pre-incubation with  $\beta$ -naphthoflavone for 24 hours induced the O-deethylation of 7-ethoxycoumarin from nearly undetectable to 140 pmol/min/mg protein in small intestine (fresh slices: 43 pmol/min/mg protein) and to 100 pmol/min/mg protein in colon slices (fresh slices: undetectable). Ketoconazole inhibited metabolism of testosterone with 40% and of 7-ethoxycoumarin with 100%. In conclusion, we showed that the intestinal slice model is an excellent model to study drug metabolism in the intestine *in vitro*, since we found that the viability parameters remain constant and the measured enzyme activities are relevant, sensitive to inhibitors and inducible. Therefore, it is a promising tool to study intestinal drug metabolism in human intestine *in vitro* in the future.

### *List of non-standard abbreviations:*

AP: Alkaline phosphatase, BNF:  $\beta$ -naphthoflavone, DMSO: Dimethylsulfoxide, KT: Ketoconazole, TOH: Hydroxytestosterone, TT: Testosterone, 7EC: 7-ethoxycoumarin, 7HC: 7-hydroxycoumarin, 7HC-GLUC: 7-hydroxycoumarin glucuronide, 7HC-SULF: 7-hydroxycoumarin sulphate

## Introduction

Although the liver has long been considered as the main drug metabolizing organ, the ability of the small intestine to metabolize drugs is increasingly recognized. Several reports have now been published showing significant contribution of intestinal tissue to the metabolism of drugs [1-3]. On the other site, no exhaustive studies to drug metabolism in the colon have been published [4].

*In vivo* assessment of intestinal metabolism is difficult due to the contribution of other organs and especially the liver to drug metabolism. Therefore, *in vitro* studies are necessary. Among the *in vitro* systems used for studying metabolism in the intestine are subcellular fractions like microsomes [5,6] and S9 fractions [7], which are commonly used due to their easy handling and applicability to human tissue. However, these systems lack membrane transporters and several enzymatic systems. Moreover, results are highly dependent on the method of their isolation [8].

Intact tissue systems, such as the everted sac preparations [9] and the Ussing chamber preparations [10], are commonly used intestinal systems in which the *in vivo* tissue structure is retained. However, the viability of these systems is open to question.

The Caco-2 cell line is also an often applied *in vitro* method for absorption studies [11]. Although Caco-2 cells during culture express many small intestinal epithelial functions drug metabolism cannot be accurately predicted by these cells [11,12].

Many factors, such as drug-drug and drug-diet interactions have been reported to influence drug metabolism and absorption in intestinal tissue by inhibition and induction [11], thereby changing the bioavailability and causing an imbalance between toxification and detoxification [13]. To date, only Caco-2 cells are available to study drug-induced induction of biotransformation in intestinal cells *in vitro* [14]. However, these cell cultures clearly differ from the complex structure and metabolic function of normal intestinal tissue. Pharmaceutical research therefore would greatly benefit from an *in vitro* system with retained intestinal drug metabolizing function that allows better prediction of drug metabolism and interactions in the small intestine of animals and humans.

Recently, a new *in vitro* model was developed in our laboratory to study drug metabolism in intact tissue of rat small intestine and colon using tissue slices. [15]. Such tissue slices show high metabolic activity towards a variety of drugs [16]. The technique has several advantages compared with the above-mentioned methods: A) It is highly efficient considering the large amount of slices that can be prepared even from small tissue samples. B) In the slices, enzyme systems, cofactors and transporters are present in their physiological context. C) Slices are relatively easy to process. These features make the intestinal slice system suitable to study metabolic properties, metabolic clearance and interactions between diet components and drugs, with respect to metabolism and transporters in animals and man.

In the present study, we characterized the viability of the intestinal slices in more detail by evaluating the morphology, with special regard to the maintenance of the mucus layer during three hours of incubation, which may be important to maintain the physiological microclimate

for the epithelial cells. In addition, the rate of drug metabolism reactions was assessed during the first three hours of incubation using testosterone (TT) and 7-ethoxycoumarin (7EC) as substrates to evaluate phase I metabolism and 7-hydroxycoumarin (7HC) as a substrate to evaluate phase II reactions. Subsequently, the metabolic rate of the three model compounds along the intestinal tract was studied. To investigate to which extent the precision-cut slice model is suitable to study drug interactions, inhibition of 7EC and TT metabolism by ketoconazole (KT) was studied. Furthermore, drug-mediated induction of 7EC metabolism was investigated in slices of small intestine and colon after 24 hours of incubation with  $\beta$ -naphthoflavone ( $\beta$ NF).

## Materials and methods

### Chemicals

6 $\beta$ -, 14 $\alpha$ -, 15 $\alpha$ -, 11 $\beta$ -hydroxytestosterone (TOH), testosterone, androstenedione, 7HC, 7HC-glucuronide (7HC-GLUC), low gelling temperature agarose (type VII-A), DMSO, eosin Y solution and ketoconazole (KT) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Gentamicin, Williams medium E with glutamax-I and amphotericin B (Fungizone)-solution were obtained from Gibco (Paisley, UK). Alcian Blue, haematoxylin solution according to Mayer, 7EC,  $\beta$ NF and dextran from leuconostoc SSP (dextran-70) were obtained from Fluka Chemie (Buchs, Switzerland). 2 $\alpha$ -, 16 $\alpha$ -, 16 $\beta$ -, 19-, and 11 $\alpha$ -TOH were obtained from Steraloids Inc. (Newport RI, USA). DePeX mounting medium was purchased from BDH laboratory Supplies (Poole, England). Tissuetek 4583 was obtained from Sakura Finetek Europe BV (Zoeterwoude, The Netherlands). Acetic acid, sodium azide, sodium chloride, calcium chloride and formaldehyde solution (37%) were obtained from Merck (Darmstadt, Germany). HEPES was obtained from ICN Biomedicals, Inc. (Eschwege, Germany). 7HC-sulphate (7HC-SULF) was a kind gift from Mr. P. Mutch, GlaxoWellcome (Herts, UK). All reagents and materials were of the highest purity that is commercially available.

### Animals

Male Wistar (HsdCpb:WU) rats weighing ca. 350 g were purchased from Harlan (Horst, The Netherlands). Rats were housed in a temperature- and humidity-controlled room on a 12-h light/dark cycle with food (Harlan chow no 2018, Horst, The Netherlands or standard 'RMH' chow Hope Farms, Woerden, The Netherlands) and tap water *ad libitum*. Pilot experiments showed no significant differences in metabolic rates in intestine from rats fed with the two chows with regard to metabolism of the model substrates (unpublished observation). The animal ethical committee of the University of Groningen approved the use of animals for these experiments.

### Preparation of precision-cut slices

Under isoflurane/N<sub>2</sub>O/O<sub>2</sub> anesthesia, the small intestine and colon were excised from the rat and put in ice-cold, oxygenated Krebs-Henseleit buffer (containing 10 mM HEPES and 25 mM D-glucose, pH 7.4). Segments of 3 cm were excised at distinct distances from the stomach and subsequently flushed with ice-cold Krebs-Henseleit buffer. One side of the segment was tightly closed and the segment was filled with 3% (w/v) agarose solution in 0.9% NaCl (37°C) and then cooled in ice-cold Krebs-Henseleit buffer, allowing the agarose solution to gel. Subsequently, the filled segment was embedded in 37°C agarose solution using a pre-cooled (0°C) tissue embedding Unit (Alabama R&D, Munford, AL USA). After the

agarose solution had gelled, precision-cut slices (thickness about 300  $\mu\text{m}$  and weight about 2 mg) were cut using a Krumdieck tissue slicer as described before [15].

### Incubation of precision-cut slices

The slices were incubated individually in a 6-wells-culture-plate (Greiner bio-one GmbH, Frickenhausen, Austria) in 3.2 ml Williams Medium E (with Glutamax-I), supplemented with D-glucose (final concentration 25 mM), gentamicin (final concentration 50  $\mu\text{g/ml}$ ) and amphotericin B (final concentration 2.5  $\mu\text{g/ml}$ ). The culture plates were placed in a pre-warmed cabinet (37°C) in plastic boxes. Slices were incubated under humidified carbogen (95%  $\text{O}_2$  and 5%  $\text{CO}_2$ ) and shaken back and forth 90 times per minute.

### Viability testing

**Histomorphology:** Precision-cut slices were incubated for 0 and 3 hours. After incubation, slices were embedded in tissuetek, snap-frozen in isopentane (-80°C) and stored at -80°C until further usage. Cryostat sections of 5  $\mu\text{m}$  were cut, fixed in formalin macrodex (0.9% NaCl, 5.4% dextran-70, 4% formaldehyde and 1%  $\text{CaCl}_2$  solution at pH 7.4) for 10 minutes at 4°C and rinsed in mQ-water. Subsequently, the sections were stained with 1% alcian blue solution in 3% acetic acid (8 min) and counterstained with haematoxylin (15 min), washed with tap water, incubated with eosin Y solution (2 min), dehydrated and embedded in DePeX mounting medium.

**Stability of drug metabolism rate:** Slices were prepared from small intestine (25-40 cm from the stomach) and colon and subsequently incubated in triplicate for 15, 30, 60 and 180 minutes with TT (final conc. 250  $\mu\text{M}$ ), 7EC (final conc. 500  $\mu\text{M}$ ) or 7HC (final conc. 500  $\mu\text{M}$ ) by addition of 32  $\mu\text{l}$  of a 100x stock solution in methanol to the 3.2 ml medium ( $n=4-6$ ). As controls, slices were incubated under standard incubation conditions without substrate and substrates were incubated without slices. After TT incubation, slice and medium were collected together and stored at -20°C until further use. After thawing (at 4°C), the slices were homogenized for 15 seconds using a sonicator (Sonicmaterials Danbury, Connecticut, USA). Subsequently, 10  $\mu\text{l}$  (1 mg/ml) 11 $\beta$ -TOH was added as an internal standard followed by addition of 6 ml methanol to precipitate the proteins. The total mixture was vortexed and centrifuged using a Beckman CS-6KR centrifuge (10 min; 4°C; at 800 x g). The supernatant was evaporated under  $\text{N}_2$  (g) and the residue was dissolved in 500  $\mu\text{l}$  50% methanol and stored at -20°C until further analysis. Just before analysis, samples were centrifuged using an Eppendorf centrifuge 5415R during 5 min (4°C; 16,000 x g) and then analyzed by HPLC as described earlier [17], using testosterone and known metabolites as a reference.

As it was previously shown that 7EC and 7HC and their metabolites are not significantly retained in the tissue, analysis was performed on medium samples only [15]. During 7EC and 7HC incubations, medium samples (200  $\mu\text{l}$ ) were harvested after 15, 30, 60, and 180 minutes and stored at -20°C until further use. After thawing, sodium azide (final conc. 1 mg/ml) was added to inhibit bacterial growth during the analysis and centrifuged using an Eppendorf centrifuge 5415R for 5 minutes at 4°C; at 16,000 x g. 7EC, 7HC, 7HC-GLUC and 7HC-SULF were used as reference. Analysis of 7EC, 7HC and their metabolites was performed using an HPLC method as described before [18]. Total 7EC metabolism, which occurs via phase I metabolism and subsequent conjugation with either glucuronide or sulphate, was calculated from the total 7HC, 7HC-GLUC and 7HC-SULF formation. All experiments were performed in 4-6 rats in triplicate.

### **Assessment of metabolic rate along the length of the intestine and colon**

Precision-cut slices were prepared from small intestine at 5, 15, 25, 40, 60, 80 cm and the last 3 cm (ileum) from the stomach and from 3 colon segments, equal in length (about 5 cm). The slices were incubated in triplicate with 7EC (final conc. 500  $\mu$ M), 7HC (final conc. 500  $\mu$ M) or TT (final conc. 250  $\mu$ M) for 3 hours and analyzed as described above. Within one experiment, tissue of one animal was used to assess the metabolic activity along the small intestine and colon of both 7EC, 7HC as TT. Each experiment was performed in 7 rats using three slices of each incubation procedure.

### **Inhibition studies**

Precision-cut slices were prepared from small intestine (at 25-40 cm from the stomach) and pre-incubated for 1 hour with or without the inhibitor ketoconazole (50  $\mu$ M). Subsequently, TT (final conc. 250  $\mu$ M) or 7EC (final conc. 500  $\mu$ M) was added and slice incubation was prolonged for 3 hours under standard conditions. Samples were harvested and analyzed as described above. Each experiment was performed in 3 rats using three slices of each incubation procedure.

### **Induction studies**

Precision-cut slices were prepared from small intestine (at 25-40 cm from the stomach) and colon and incubated with  $\beta$ NF (final conc. 50  $\mu$ M or 100  $\mu$ M, added as a 100-times concentrated stock solution in DMSO) for 24 hours under standard incubation conditions as described in the section: incubation of precision-cut slices. Control slices were incubated in medium with DMSO but without  $\beta$ NF. After 24 hours of incubation, slices were transferred to fresh medium and incubated for 3 hours with 7EC (final conc. 500  $\mu$ M) to assess metabolic activity. Samples were analyzed as described above. Each experiment was performed in 3 rats in triplicate.

### **Protein determination**

After incubation with 7EC or 7HC, slices were stored at  $-20^{\circ}\text{C}$  until further use. After thawing, 20  $\mu$ l 5 N NaOH was added to the slice followed by 40 minutes of incubation at  $37^{\circ}\text{C}$  to dissolve the tissue. 980  $\mu$ l water was added to dilute the NaOH concentration to 0.1 M, after which the mixture was homogenized by 5 seconds of sonication. Samples were diluted and the protein content was determined using Bio-Rad protein assay dye reagent (Bio-Rad, Munich, Germany) using BSA as standard. After testosterone incubation, the protein was not determined. For these slices, the average protein contents of comparable incubations with 7EC and 7HC within the same experiment were used.

### **Liver slices**

Data on liver slice metabolism were taken from earlier studies [19].

### **Statistics**

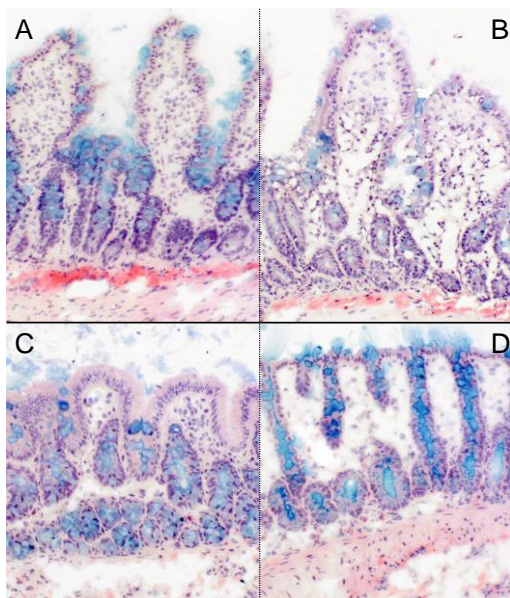
Statistical significance was determined using Student's *t*-test.

## Results

### Viability of intestine and colon slices

Two different parameters were evaluated after 3 hours of incubation to assess the viability of small intestinal and colon slices.

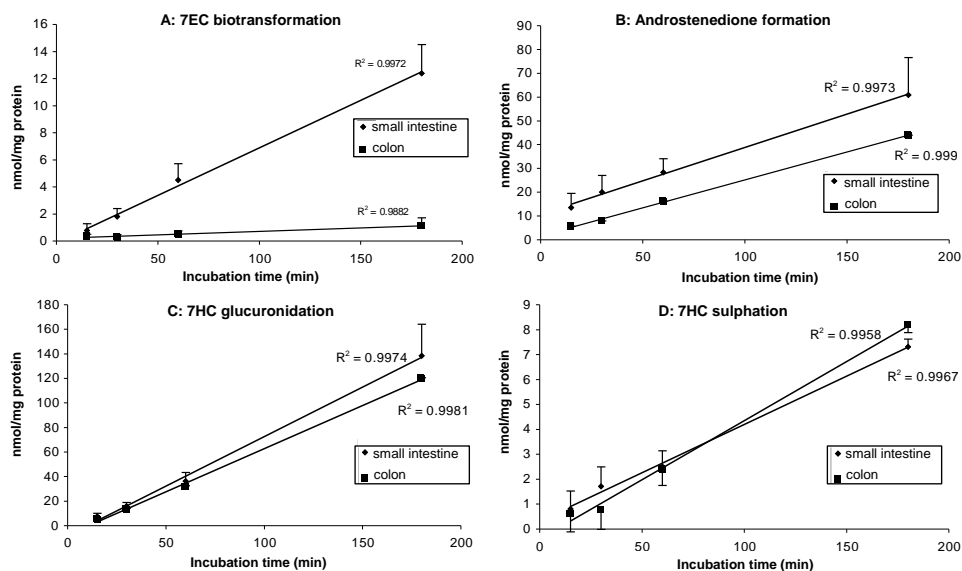
**Histomorphology:** Viability of colon and small intestinal slices was assessed by histomorphological evaluation. Alcian blue staining indicated that goblet cells still contain mucus and the mucus layer was partly retained after preparation and after 3 hours of incubation. In addition, epithelial cells showed intact morphology, but villi of small intestine appeared somewhat flattened/broadened during incubation (figure 1).



**Figure 1:** Morphological evaluation of slice preparations of the small intestine after 0 hours (A) and 3 hours (B) of incubation and colon slices after 0 (C) and 3 hours (D) of incubation. Staining: Alcian Blue counterstained with HE (100x)

**Stability of drug metabolism rate:** Figure 2 shows the stability of metabolic rates of 7EC, TT (phase I substrates) or 7HC (phase II substrate) during the first 3 hours of incubation in slices prepared from small intestine and colon. A time-dependent linear formation rate of metabolites was observed during 3 hours of incubation for all compounds tested in both small intestine ( $R^2 > 0.99$ ) and colon ( $R^2 > 0.98$ ). 7EC metabolism in colon was low and in some experiments below the detection level. After incubation with testosterone, only androstenedione formation was detected both in small intestine and colon.





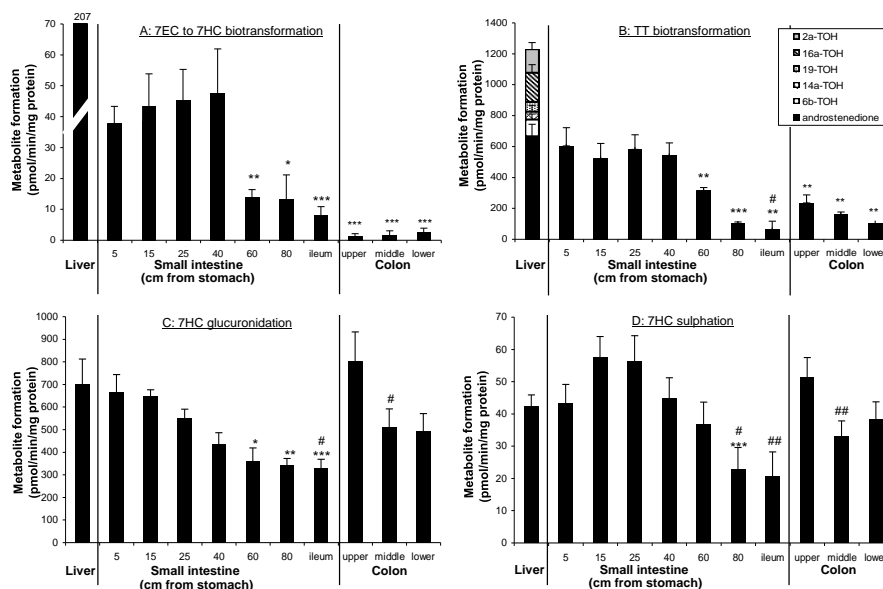
**Figure 2:** Metabolizing activity of precision-cut slices prepared from small intestine (at 25-40 cm from the stomach) (◆) and colon (■) during 3 hours of incubation under standard conditions: 7EC into 7HC (A); testosterone into androstenedione (B); 7HC into 7HC-GLUC (C) and 7HC-SULF (D). Results are mean  $\pm$  SEM of 4-6 rats; in each experiment at least 3 slices were incubated per time point.

### Assessment of metabolic rate along the length of the intestine

The metabolic activity of model compounds was assessed in slices from small intestine and colon excised at distinct distances from the stomach. Activities showed a decreasing gradient along the small intestine towards colon, with the slope of the gradient depending on the model compound used.

O-deethylation (mediated by CYP1A and 1B) of 7EC was constant and highest in the first 40 cm of the proximal site of the small intestine (about 40 pmol/min/mg protein or 4.4 nmol/min/g wet weight as the intestinal protein content appeared 109 mg/g wet weight) (figure 3A) after which the metabolic rates gradually and significantly decreased (5, 15 and 25 cm towards 60-80-ileum cm,  $p < 0.05$ ) to 8 pmol/min/mg protein (or 0.9 nmol/min/g wet weight) in ileum (figure 3A).

Although 7EC O-deethylation in colon slices was below detection level in some experiments and in others significantly lower compared with the proximal part of the small intestine ( $p < 0.001$ ), the average metabolic rate in colon (about 2 pmol/min/mg protein or 0.2 nmol/min/g wet weight) was non-significantly lower than in the distal part of the small intestine. The increase in metabolic rate in colon in distal direction was not significant. 7EC O-deethylation was significantly ( $p < 0.01$ ) lower in small intestine (4-23%) and colon (1%) compared to liver slices (207 pmol/min/mg protein [19] or 22 nmol/min/g wet weight).



**Figure 3:** Metabolic activity along the intestinal tract. Precision-cut slices from small intestine were prepared at distinct distances from the stomach, incubated for 3 hours with 7EC (A), testosterone (B) or 7HC (C and D). Phase II metabolism of 7HC is shown in two different graphs: the formation of 7HC-GLUC (C) and 7HC-SULF (D). Results are mean  $\pm$  SEM of 7 rats. Significant differences toward small intestine (at 5 cm) are indicated with \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ; Significant differences between upper colon and ileum, middle and lower colon were tested and indicated with #  $p < 0.05$ , ##  $p < 0.01$ , ###  $p < 0.001$ .

To compare the activity of the metabolically active cells in liver and intestine (hepatocytes and epithelial cells), metabolic activity was recalculated per mg hepatocyte or epithelial protein. Due to lack of data, activity is expressed per epithelial protein and not per enterocyte protein despite the fact that enterocytes are the metabolically active epithelial cells. Thus, taking into account that small intestine contains 18.1 mg epithelial protein per g wet weight [20] and our slices contained 109 mg protein per g wet weight, the specific activity per mg epithelial protein is  $109/18.1 = 6$  times the activity per mg small intestinal protein. Due to lack of data, the same factor is used for colon. Likewise, with liver, about 80% of liver protein is hepatocyte protein. Assuming that roughly 50% of the hepatocytes in the slice contribute to the metabolic activity (unpublished research): the activity per mg hepatocyte is  $2 \times 100/80 = 2.5$ .

The metabolic activities of 7EC O-deethylation in epithelial cells of small intestine and colon were respectively 4-26 and 1.3 nmol/min/g epithelial cell, which is 7-47% respectively 2.4% of the hepatocyte activity (55 nmol/min/g hepatocyte).

Androstenedione was the only metabolite that could be detected after incubation of slices with TT and appeared to be formed at the highest rate in the first 40 cm of the proximal site of the small intestine (about 550 pmol/min/mg total protein or 60 nmol/min/g total wet weight) (figure 3B) and decreased significantly ( $p < 0.01$ ) in distal direction to 61 pmol/min/mg

protein (or 7 nmol/min/g wet weight). In colon, the metabolic rate of androstenedione formation was significantly lower (232-105 pmol/min/mg protein or 25-11 nmol/min/g wet weight) compared with the proximal part of the small intestine ( $p < 0.01$ ), but significantly higher compared with the distal part of the small intestine ( $p < 0.02$ ) and also tended to decline in distal direction. The activity found in proximal part of small intestine was comparable to liver (666 pmol/min/mg total protein [19] or 72 nmol/min/g total wet weight). On the other hand, colon (16-35%) and the distal part of the small intestine (11%) showed significantly lower specific activity compared with liver. CYP mediated reactions, such as 6 $\beta$ -, 15 $\alpha$ -, 19-, 16 $\alpha$ -, 16 $\beta$ -, and 2 $\alpha$ -TOH formation were not detectable in both small intestine and colon. These metabolites were readily detected using liver slices in rates up to 563 pmol/min/mg protein, resulting in a total metabolic rate in liver for testosterone of 1,230 pmol/min/mg protein (or 131 nmol/min/g wet weight). Converted to the amount of active cells, the metabolic rates toward testosterone per epithelial cells in small intestine (proximal: 360 and distal: 42 nmol/min/g epithelial cell) were comparable or higher compared with liver (328 nmol/min/g hepatocytes).

Phase II metabolism was assessed by measuring 7HC biotransformation. The glucuronidation rate was high in the proximal part of the small intestine (663 pmol/min/mg protein or 72 nmol/min/g wet weight) (figure 3C) and decreased significantly only after 60 cm ( $p < 0.05$ ) in distal direction to 326 pmol/min/mg protein (or 36 nmol/min/g wet weight). Subsequently, in the proximal part of colon, the metabolic rate increased again to even higher rates (800 pmol/min/mg protein or 87 nmol/min/g wet weight) compared with the distal part of the small intestine and decreased significantly ( $p < 0.05$ ) to 490 pmol/min/mg protein (or 53 nmol/min/g wet weight) distally. Glucuronidation rates in both small intestine and colon slices were comparable to rates measured in liver slices (700 pmol/min/mg protein [19] or 74 nmol/min/g wet weight) being 49-97% and 72-118% respectively. Converted to the specific activity of epithelial cells in small intestine (214-435 nmol/min/g epithelial cell) and colon (322-525 nmol/min/g epithelial cell), the activity was 1-3 times higher activity compared with hepatocytes (186 nmol/min/g hepatocyte).

Sulphation of 7HC (figure 3D) showed a different pattern along the small intestine than other metabolic pathways: the metabolic rate initially increases non-significantly from 43 pmol/min/mg protein (4.7 nmol/min/g wet weight) to 58 pmol/min/mg protein (or 6 nmol/min/g wet weight) with the highest metabolic rate measured at 15 cm from the stomach. The metabolic rates decreased significantly from 40 cm onward to a final rate of 21 pmol/min/mg protein (2 nmol/min/g wet weight) in ileum ( $p < 0.05$ ). In colon, the proximal metabolic rate was significantly higher than in the distal part of the small intestine ( $p < 0.05$ ). A gradient decreasing from 51 to 38 pmol/min/mg protein (or 6 to 4 nmol/min/g wet weight) was shown in distal direction. Specific metabolic activities in the small intestine and colon appeared to be equal or even higher than those of liver slices (42 pmol/min/mg protein [19] or 4.5 nmol/min/g wet weight), being 50-140% and 90-120% of liver slice values. When converted to sulphation activity per amount of active cells, the cells in small intestine (14-38 nmol/min/g epithelial cell) and colon (25-34 nmol/min/g epithelial cell) have 1-3 times higher activity compared with hepatocytes (11 nmol/min/g hepatocyte).

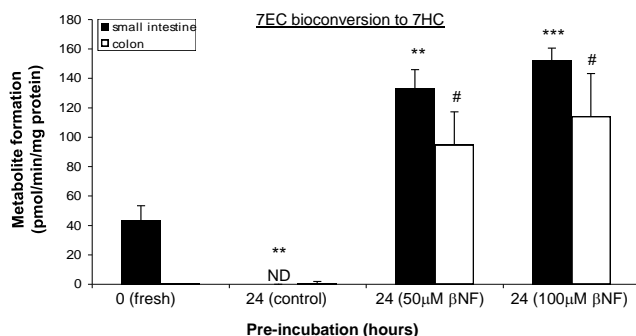
## Inhibition studies

Slices were pre-incubated with KT for 1 hour followed by 3 hours of incubation with TT or 7EC. TT metabolism was only partially inhibited to  $61 \pm 4\%$  (from  $432.6 \pm 194$  to  $255.3 \pm 89$  pmol/min/mg protein); in contrast 7EC metabolism was totally blocked (from  $22.7 \pm 6.3$  pmol/min/mg protein). Results are mean  $\pm$  SEM of 3 rats; in each experiment 3 slices were incubated per treatment.

## Induction studies

Induction of 7EC metabolism in precision-cut slices of small intestine and colon was measured after 24 hours of incubation with  $\beta$ NF (figure 4). A very strong induction of phase I metabolism of 7EC was found in both small intestine and colon. Directly after slicing, the metabolic rate of 7EC in slices prepared from small intestine was about 43 pmol/min/mg protein ('fresh slices'), but declined to nearly undetectable levels after 24 hours of pre-incubation in control medium without  $\beta$ NF ( $p < 0.01$ ). In contrast, when small intestinal slices were pre-incubated with  $\beta$ NF for 24 hours, the metabolic rate increased strongly to 140 pmol/min/mg protein ( $p < 0.001$  compared with fresh slices). The increase was independent of the  $\beta$ NF concentrations used; both concentrations (50  $\mu$ M and 100  $\mu$ M) showed a 3.5-fold induction of the metabolic rate compared with the rate of fresh slices.

In colon slices, phase I metabolism of 7EC could be induced by  $\beta$ NF as well. 7EC O-deethylation of colon slices was near the detection level in both fresh slices and slices after 24 hours of pre-incubation. However, when incubated for 24 hours with  $\beta$ NF, the metabolic rate increased tremendously to about 100 pmol/min/mg protein ( $p < 0.05$ ).



**Figure 4:** Induction of 7EC metabolism in precision-cut slices prepared from small intestine (25-40 cm from stomach) (black columns) and colon (open columns). Slices were incubated for 24 hours with 0, 50 or 100  $\mu$ M  $\beta$ -naphthoflavone ( $\beta$ NF) transferred to fresh medium and incubated with 500  $\mu$ M 7EC for 3 hours. Activity in fresh slices was determined directly after slicing without pre-incubation. (ND is non-detectable.) Results are mean  $\pm$  SEM of 3 rats. In each experiment 3 slices were incubated per treatment. Significant differences toward the activities of fresh small intestinal slices are indicated with \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ . Significant differences in colon metabolic activity toward fresh colon are indicated with #  $p < 0.05$ , ##  $p < 0.01$ , ###  $p < 0.001$ .

## Discussion

Recently, we presented rat small intestinal and colon precision-cut slices as a tool to study intestinal metabolism [15]. In this study, we further characterized the viability and functionality of these slices. We assessed the gradient of several phase I and phase II enzyme activities along the intestine, as well as the applicability of slices to investigate drug interactions.

The metabolic rate of model compounds (7EC, 7HC or TT) was constant during the first three hours of incubation. In addition, hardly any signs of degradation of colon and intestinal slices were observed upon histomorphological examination. Moreover, the mucus layer which presumably protects the epithelial lining *in vivo* [21] remained present. Altogether, these results imply the integrity of slices during three hours of incubation.

The intestine is not homogenous with respect to drug metabolism along its length [6] nor along the villi [22]. Generally, a decreasing gradient in distal direction is reported [1]. However, the preparation method of intestinal tissue has been found to be critical for the outcome of quantitative evaluation of intestinal metabolism [8]. Gradients of mRNA expression only allow speculation of protein expression gradients, whereas Western blotting and immunohistochemical staining of metabolizing proteins reveal only the enzyme distribution pattern along the tract. This might not necessarily correlate with the *in vivo* distribution of the metabolic activity, since other factors like cofactors and transporters can be rate-limiting. Tissue slices may be pre-eminently suitable to study the distribution of metabolic activity along the intestine, since no isolation steps are required. In addition, cofactors and transporters are supposed to be present at physiological levels.

Gradients of metabolic activity differed depending on the substrate used. Metabolic activity towards 7EC (CYP1A and 2B) was the highest in the proximal part of the small intestine, which is in accordance with the reported higher levels of CYP1A1 and 2B1 protein in duodenum compared with ileum [23]. However, others described an initial increase followed by a decrease in metabolic activity towards 7EC in distal direction along the intestinal tract [24]. To our knowledge, data describing the 7EC metabolism gradient in colon have not been published before.

In rat, testosterone is metabolized by several CYP-isozymes such as CYP1A1/2, 2C11, 3A1/2 [25] and by 17 $\beta$ -hydroxysteroid dehydrogenase (17 $\beta$ -HSD) [26]. Androstenedione formation is mediated by 17 $\beta$ -HSD and several CYP-isozymes [7] as well. This was confirmed in the present study, where KT inhibited androstenedione formation with 40%. KT totally blocked 7EC metabolism as expected. In addition, the gradient of androstenedione formation found appeared to be similar to the 17 $\beta$ -HSD activity [26] along the length of the intestine, indicating a large contribution of 17 $\beta$ -HSD to androstenedione formation. An increase of androstenedione formation in distal direction including colon using S9-fractions was reported by others [7]. However, these activity rates were over 100-fold lower (0.35 nmol/min/g intestine) than ours, impeding good comparison. Besides androstenedione, no other metabolites of TT were found in the present study, contrary to the findings of Takemoto et al. [6], who found considerable 6 $\beta$ -hydroxytestosterone formation (CYP3A). However, others reported non-detectable levels of CYP3A1 mRNA [27] or low levels of 6 $\beta$ -

hydroxytestosterone formation [7] in intestinal preparations. We found that when 4 slices per well were incubated, 6 $\beta$ -hydroxytestosterone formation was detected (unpublished observation). This indicates that CYP3A activity is present, but undetectable due to the small amount of tissue used.

Phase II metabolism along the intestine was assessed using 7HC as a substrate, which is conjugated. For 7HC-GLUC formation, we observed a decreasing gradient in distal direction. Sulphation, thought to be important in overall metabolism in small intestine, especially in comparison with total liver capacity [24], showed a small, but non-significant increase followed by a decrease.

The underlying cause of the gradients is unknown. A metabolic gradient could be explained by a gradient in the number of metabolizing cells and/or in enzyme activity per enterocyte along the intestine. The latter can be caused by enterocyte differences in enzyme expression, which may be induced by gradients of transcription factors or inducing components, or by differences in the presence of transporters. The number of enterocytes alone cannot explain the gradients, since activity patterns differed for the substrates used.

The rate of 7EC O-deethylation in proximal small intestine (4.4 nmol/min/g intestine) is over 100-fold higher than reported by others (10.3 and 16.6 pmol/min/mg microsomal protein in mucosa, which corresponds to 25 and 40 pmol/min/g intestine) using respectively microsomes and everted sacs of proximal small intestine [6]. As microsomal activities are measured under optimal cofactor conditions, one would expect at least equal or higher activities in microsomes compared with intact cells where cofactor concentrations may be suboptimal. Possibly the lower activity found with microsomes is caused by enzyme inactivation by proteases or their low isolation yield, which does not play a role when slices are used.

Small intestinal specific activity per total protein was about 4-23% of the hepatic 7EC O-deethylation, confirming the results of Shirkey et al. with microsomes [28]. However, when expressed per metabolically active epithelial cells, the activity is estimated to amount 8-47% of hepatocytes, indicating the high metabolic competence of epithelial cells.

The metabolic activities toward TT found in our system (proximal: 360 and distal: 42 nmol/min/g epithelial cell) are comparable with the androstenedione formation rates in scraped mucosa homogenates: 595-29-157 nmol/min/g intestinal mucosa (duodenum-ileum-colon) reported by others [26]. In slices, epithelial cells of small intestine and colon are estimated to process androstenedione formation about 0.2-2 times faster than hepatocytes. However, the competence of colon epithelial cells is probably underestimated, since the scaling factor for small intestinal epithelial protein is used due to lack of colon data. The colon epithelial surface area is smaller than that of small intestine, whereas the muscle layer in colon is also thicker. In contrast, Sohlenius-Sternbeck et al. found in small intestinal S9-fractions only 0.9-5.2% of the androstenedione formation measured in liver [7].

7HC glucuronidation (36-72 nmol/min/g intestine) and sulphation in slices (2-6 nmol/min/g intestine) is about 1-5 times higher than in isolated epithelial cells (13.1 and 2.3 nmol/min/g intestine [20]). Interestingly, the rates of glucuronidation and sulphation in small intestinal and colon epithelial cells were estimated to be 1-3 times higher than the estimated rates of hepatocytes measured in liver slices.

The high metabolic rate in intestinal epithelial cells in comparison with the hepatocytes indicates the important role of the intestine in drug metabolism, its ability to contribute significantly to the *in vivo* first-pass metabolism and the risk of site-specific toxicity caused by toxic metabolites formed in the gut.

Up till now, an adequate *in vitro* model to study enzyme induction in the intestine was lacking. To mimic enzyme induction *in vitro*, intact cellular machinery is required. Existing *in vitro* systems such as everted sacs and Ussing Chambers have never been proven to survive long enough to allow incubation with an inducer for 24 hours.

In the present study, we found a clear induction of the metabolic activity of slices towards 7EC after incubation for 24 hours with  $\beta$ NF (induction via aryl hydrocarbon receptor [13]). Administered *in vivo*,  $\beta$ NF was reported to significantly induce CYP1A1 mRNA, protein and activity [29] and 7EC O-deethylation in small intestine [30] and colon [31]. Our finding is also consistent with the induction of CYP1A1/1A2 and 2B1 mRNA by  $\beta$ NF in intestinal slices reported by Martignoni et al. [27].

These data, showing a prominent induction of metabolic activity in slices after 24 hours of incubation, are a strong indication that the low basal metabolism of 7-EC after 24 hours of pre-incubation cannot be due to loss of viability only. After all, viability is indirectly indicated by inducibility and by a metabolic activity after induction being much higher than in the control slices at the first 3 hours of incubation. This decrease may rather indicate some kind of down-regulation, due to a lack of physiological stimuli, or to the loss of epithelial cells during incubation. Further study is required to confirm this hypothesis.

In conclusion, the present study has shown that the intestinal slice model can serve as a tool to study drug metabolism in the intestine *in vitro*, since it was stable during at least 3 hours and comparable or even higher metabolic activities than described for other methods were observed. In addition, the model is able to detect gradients of metabolic activity along the intestine, inhibitory drug-drug interactions and induction responses. We conclude that the slice model is a promising tool to study these phenomena in small intestine and colon *in vitro*, also in human tissue in future.

### Acknowledgements

Dr J Nieken is acknowledged for help with histomorphological assessment, J Visser for help with HPLC analysis and A van Loenen-Weemaes for help with animal handling.

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